

Determination of PQQ in quinoproteins with covalently bound cofactor and in PQQ-derivatives

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Application of the so-called hexanol extraction procedure for PQQ determination, originally based on detachment of the cofactor from quinoproteins and conversion into PQQ-5,5-dihexyl ketal, leads in several cases to a number of products due to uncontrollable esterification. The present modified procedure, detaching the covalently bound cofactor and converting it into 4-hydroxy-5-hexoxy-pyrroloquinoline, was tested on a number of proteins. Only the expected product was obtained for the known quinoproteins, in a quantitative yield, as revealed by comparison with the values determined with the hydrazine method. Thus this independent method confirmed that bovine serum amine oxidase, porcine kidney diamine oxidase, dopamine β -hydroxylase from bovine adrenal medulla, methylamine dehydrogenase from *Thiobacillus versutus*, glutamate decarboxylase from *Escherichia coli*, and 3,4-dihydroxyphenylalanine decarboxylase from pig kidney are really quinoproteins. Quantitative conversion was also achieved for condensation and addition products of PQQ (PQQ-acetone, PQQH₂, PQQ-oxazole, PQQ-dinitrophenylhydrazone, and PQQ-tryptophan). In view of this conversion and the fact that catalytic activity of PQQ is not required, the method seems suited to investigate the distribution of the cofactor in eukaryotes, especially in mammals where it is almost certain that PQQ occurs only in derivatized form. Finally, just like the hydrazine method, the hexanol extraction procedure seems unable to keep the structure of the cofactor as it exists in the active site, intact, as demonstrated for the pro-PQQ cofactor of methylamine dehydrogenase.

Pyrroloquinoline quinone; Quinoprotein; Cofactor; Hydrazone

1. INTRODUCTION

The hydrazine method [1] has been a powerful tool in establishing the quinoprotein nature of a large number of enzymes where the cofactor occurs in a covalently bound form (see [2]). However, it can be imagined that it will fail in certain cases, for instance if the enzyme-bound cofactor is not in the oxidized state, if the active site does not allow reaction of pyrroloquinoline quinone (PQQ) with the hydrazine, or the cofactor is present in the form of a condensation product or an adduct. In this respect, a precedent already exists, namely 3,4-di-

hydroxyphenylalanine (dopa) decarboxylase from pig kidney, where derivatization of the cofactor is limited to only a few percent [3].

Tyrosine and glutamic acid are the precursors for biosynthesis of free PQQ in methylotrophic bacteria [4,5]. Synthesis of covalently bound PQQ occurs in organisms which do not produce the free form, as demonstrated for glutamate decarboxylase from *Escherichia coli* [6]. This also applies to eukaryotes: free PQQ has not been detected but several enzymes have covalently bound PQQ, suggesting that synthesis of their cofactor occurs in situ [4,7]. On the other hand, mammalian organisms have PQQ in their food (e.g. vinegar contains substantial amounts of free PQQ and it is likely that proteolysis of quinoproteins in the digestive tract detaches the cofactor) and positive

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effects have been noted on administration of the compound to the diet of rats [8]. Thus, it is still interesting to trace the uptake and distribution of the compound and to elucidate its role in mammalian organisms. However, in view of the reactivity of PQQ towards nucleophilic compounds, analysis is problematic. For instance, PQQ reacts with amino acids to the so-called oxazoles [9], products which are inactive in a biological assay with a quinoprotein dehydrogenase apo-enzyme and which may occur in a variety of presently indeterminable forms. Thus, to solve this problem, a method is required which quantitatively converts all condensation and addition products to either PQQ or a well-defined derivative.

In view of the possible drawbacks indicated of the hydrazine method, the attractiveness of an independent second method and the need for a procedure able to analyze PQQ in its derivatized forms, the so-called hexanol extraction procedure was developed. In this procedure, the sample is refluxed with a mixture of HCl and hexanol so that the dihexyl ketal of PQQ is formed. It has already been applied in the case of 3,4-dihydroxyphenylalanine (dopa) decarboxylase from pig kidney [3], and galactose oxidase from *Dactylium dendroides* [10]. Originally, the rationale behind this method was the following: the higher aliphatic alcohol, *n*-hexanol, adds to PQQ, forming a ketal, and provides a high temperature during refluxing so that hydrolysis with HCl proceeds in an efficient way; the protonated ketal is soluble in the organic layer, escaping unwanted attack by other nucleophilic compounds (e.g. amino acids detached from the protein). However, an unattractive aspect of the procedure is that sometimes uncontrolled esterification of the ketal occurs, as demonstrated in the case of dopa decarboxylase [3].

Addition compounds like hemiketals and ketals are inherently unstable but can be transformed by reduction and dehydration into the very stable 4-hydroxy-5-alkoxy-pyrroloquinoline compounds [11]. It appeared that upon changing the conditions of the hexanol extraction procedure, the conversion of the ketal into 4-hydroxy-5-hexoxy-pyrroloquinoline (4-hydroxy-5-hexoxy-PQ) could be achieved directly and esterification could be avoided. The present paper demonstrates the reliability with respect to detection and quantification of the modified procedure.

2. MATERIALS AND METHODS

2.1. Chemicals

PQQ was synthesized using the method of Corey and Tramontano [12], as revised by Jongejan [13]. The acetone adduct of PQQ [14], PQQH₂ [15], PQQ-oxazole from glycine [9], the hydrazone of PQQ and 2,4-dinitrophenylhydrazine [16], and a PQQ-tryptophan condensation product [17] were prepared as described. Bovine serum albumin was from Sigma (crystallized and lyophilized, product no. A7511). Hexanol (>98%, product no. 804393) and all other chemicals were from Merck (Darmstadt, FRG). Sep-Pak C₁₈ cartridges were from Waters.

2.2. High-performance liquid chromatography

HPLC occurred on a 5 μ m reversed-phase C₁₈-RCM cartridge (8.0 \times 100 mm), equilibrated with 10 mM sodium acetate (pH 4.5), in a RCM-100 module (Waters). The eluent consisted of a 20 min linear gradient (flow rate 1.5 ml/min) from 10 mM sodium acetate (pH 4.5) to 10 mM sodium acetate (pH 4.5)/acetonitrile (40:60, v/v), holding the final concentration for 5 min. Subsequently the column was recovered by applying a 5 min linear gradient from the 40:60 ratio eluent to 10 mM sodium acetate (pH 4.5).

2.3. Cofactor extraction from enzymes and condensation products of PQQ

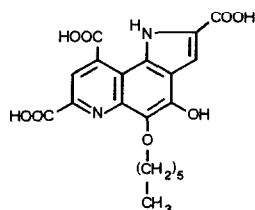
Enzymes were purified as described in the references given in table 1.

To a 7.5 ml sample of enzyme (0.5–35 mg protein in 0.2 M potassium phosphate, pH 7.0) or condensation product of PQQ (1 μ mol in water), 7.5 ml of 6 M HCl and 10 ml *n*-hexanol were added. After refluxing for 30 min (temperature 160°C), the water phase was removed by evaporation. After adding an additional 10 ml of *n*-hexanol, the solution was refluxed for an additional hour, after which the hexanol was evaporated under reduced pressure. Traces of hexanol were removed by keeping the residue overnight under vacuum over P₂O₅. The residue was dissolved in methanol, and the sample diluted ten times with water. Subsequently, the so-called Sep-Pak procedure was applied: the mixture was acidified to pH 2.0 with 6 M HCl and applied to a Sep-Pak C₁₈ cartridge (in portions of 10 ml); the cartridge was washed with 10 mM HCl, subsequently with water, and the product eluted with 1 ml methanol. Quantification of PQQ was performed by measuring the amount and absorbance of the eluate and using the molar absorption coefficient of compound 1 ($\epsilon_{318\text{ nm}} = 39\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$). Homogeneity and identity of the extracted product were checked by HPLC with photodiode array detection.

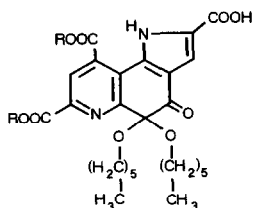
2.4. Preparation of PQQ-5,5-dihexyl ketal and its dihexyl ester

100 mg PQQ, dissolved in 150 ml of 3 M HCl, was mixed with 50 ml *n*-hexanol. The mixture was refluxed for 2 h. After cooling to room temperature, the hexanol layer was removed and washed with 200 ml portions of water until neutral. The hexanol was evaporated under reduced pressure. Traces of hexanol were removed by keeping the residue overnight under vacuum over P₂O₅ (yield, 85 mg of compound 2).

A further reaction with hexanol took place when refluxing with hexanol was allowed to proceed for an additional 4 h. This was achieved in a separate experiment where 25 mg PQQ,



(1) 4-hydroxy-5-hexoxy-PQ



(2) PQQ-5,5-diethyl ketal (R=H)

(3) diethyl ester of PQQ-5,5-diethyl ketal (R=C₆H₁₃)

Scheme 1. Structures of 4-hydroxy-5-hexoxy-PQ, PQQ-5,5-diethyl ketal and its diethyl ester.

dissolved in 40 ml of 3 M HCl, was mixed with 15 ml *n*-hexanol. After refluxing for 6 h, the product (compound 3, yield 23 mg) was isolated as described for compound 1 (yield 23 mg).

2.5. Conversion of PQQ-5,5-diethyl ketal into 4-hydroxy-5-hexoxy-PQ

Reduction was performed by dissolving 40 mg of compound 2 in 50 ml water, flushing the solution with Argon for 15 min, after which 40 mg NaBH₄ was added. Completion of the reaction (after approx. 2 h) was checked by HPLC. Product was recovered using the Sep-Pak procedure. Methanol was evaporated under reduced pressure (yield 36 mg). Conversion into 4-hydroxy-5-hexoxy-PQ was performed by dissolving the dried preparation (36 mg) in 10 ml conc. H₂SO₄ [11]. After 20 min at 50°C, the reaction was complete, as judged by HPLC, and the mixture diluted with water. The product (compound 1) was recovered by the Sep-Pak procedure (yield 29 mg).

2.6. Conversion of 4-hydroxy-5-hexoxy-PQ into PQQ

Conversion of compound 1 into PQQ was effected by oxidation with a cerium salt in a procedure described for *o*-quinone formation [20]. 10 mg of compound 1 was dissolved in 10 ml water, and 5 mg ammonium cerium nitrate was added. Conversion was followed by HPLC (preceded by application of the Sep-Pak procedure to the samples).

2.7. NMR spectroscopy

¹H-NMR spectra of the PQQ derivatives were recorded at 30°C with a Varian VXR 400S spectrometer operating at 400 MHz. The solvent was (C²H₅)₂SO and tetramethylsilane was used as an internal reference.

3. RESULTS

3.1. PQQ-5,5-diethyl ketal

The reaction of PQQ with hexanol in the presence of 3 M HCl yielded a product (compound 2) with an absorption spectrum (fig.1) closely resembling that of the acetone adduct of PQQ [14,21]. The preparation was practically homogeneous since the HPLC system showed the presence of only tiny amounts (less than 0.5%) of impurities, most probably hexyl-esters of compound 2.

¹H-NMR signals were observed at 0.87 (t 7 Hz, -CH₂-CH₃), 1.2–2.0 (multiplets, -(CH₂)₄-), 4.06 (t 4 Hz, -CH₂O-), 7.28 ppm (d 2 Hz, 3-H), 8.88 (s, 8-H) and 16.22 ppm (broad, 1-H). Integration of the spectrum revealed the presence of two hexyl groups per PQQ moiety. As the structure of the acetone adduct of PQQ has been unequivocally established by X-ray analysis [22], the similarity between the compounds suggests the presence of a keto group at C₄ and a disubstitution at C₅. Together with the other data, this leads to the view that compound 2 is the PQQ-5,5-diethyl ketal. Compound 2 has an $\epsilon_{357\text{ nm}}$ of 8700 M⁻¹·cm⁻¹, and its absorption spectrum is shown in fig.1.

When the refluxing was allowed to proceed for another 4 h in the absence of water, further reaction took place. The ¹H-NMR spectrum of this

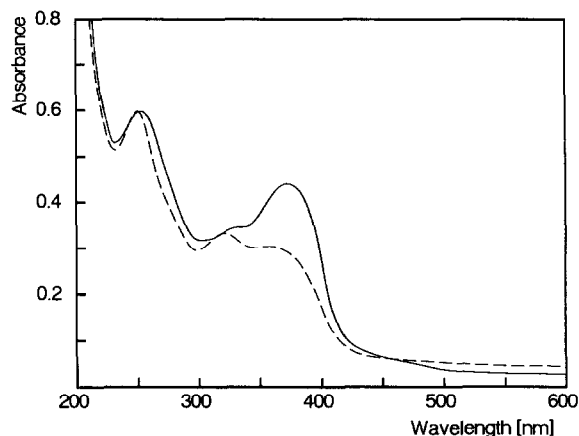


Fig.1. Absorption spectra of PQQ-5,5-diethyl ketal (compound 2), and its diethyl ester (compound 3). HPLC was performed as indicated in section 2.2. The spectra were taken in the apex of the eluting peaks with retention times of 24.3 ± 0.5 min and 6.8 ± 0.5 min for compound 2 (—) and compound 3 (---), respectively.

product (compound 3) showed signals at 0.89 (t 7 Hz, $-\text{CH}_2-\text{CH}_3$), 1.2–2.4 (multiplets, $-(\text{CH}_2)_4-$), 4.34 (t 4 Hz, $-\text{CH}_2\text{O}-$), 4.42 (t 4 Hz, $-\text{CH}_2\text{O}-$), 4.53 (t 4 Hz, $-\text{CH}_2\text{O}-$), 7.24 (d 2 Hz, 3-H), 8.60 (s, 8-H) and 14.6 ppm (broad, 1-H). Integration of the spectrum revealed the presence of 4 hexyl groups per PQQ moiety, two of which are responsible for the signal at 4.34 ppm. On the basis of these results, it is concluded that compound 3 is the PQQ-5,5-dihexyl ketal with hexyl esterification at the C_7 and C_9 position (in view of its acidity [23], the C_2-COOH is probably the most difficult one to esterify). The absorption spectrum of compound 3 is shown in fig.1.

3.2. Conversion of PQQ-5,5-dihexyl ketal into 4-hydroxy-5-hexoxy-PQ

Reduction of compound 2 with NaBH_4 , followed by dehydration in conc. H_2SO_4 , resulted in the formation of a product with an absorption spectrum (fig.2) closely resembling that of PQQH₂ [24] and 4-hydroxy quinoline derivatives of PQQ [11]. $^1\text{H-NMR}$ spectroscopy showed signals at 0.85 (t 7 Hz, $-\text{CH}_2-\text{CH}_3$), 1.4–1.9 (multiplets, $-(\text{CH}_2)_4-$), 4.30 (t 4 Hz, $-\text{CH}_2\text{O}-$), 7.20 (d 2 Hz, 3-H), 8.34 (broad, 4-OH), 8.61 (s, 8-H) and 13.0 ppm (broad, 1-H). Integration of the spectrum indicated the presence of one hexyl group per PQ moiety. Just as has been found for other 4-hydroxy quinoline

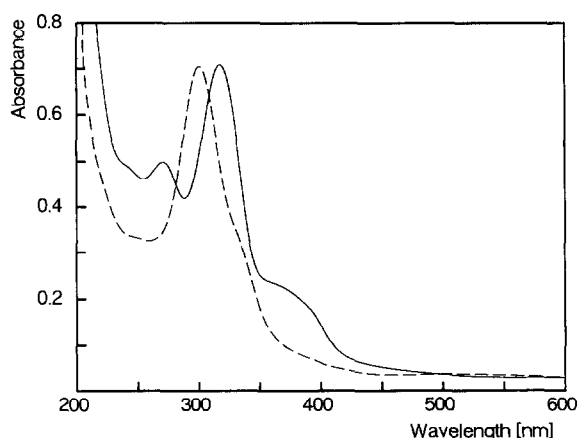


Fig.2. Absorption spectra of 4-hydroxy-5-hexoxy-PQ (compound 1) and of PQQH₂. HPLC was performed as indicated in section 2.2. The spectra were taken in the apex of the eluting peaks, showing retention times of 14.5 ± 0.5 min and 9.3 ± 0.5 min for compound 1 (—) and PQQH₂ (---), respectively.

structures [12,20], oxidation of compound 1 with the cerium salt led to the original quinone compound, as was evident from the PQQ peak present in HPLC recordings, and from appearance of activity in a biological assay (results not shown). Quantitative conversion was achieved in 1 h at room temperature (fig.3). From these results, it is concluded that the structure of compound 1 is 4-hydroxy-5-hexoxy-PQ. Compound 1 has an $\epsilon_{318 \text{ nm}}$ of $39\,900 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

3.3. The hexanol extraction procedure

3.3.1. PQQ and its condensation products

When the previous hexanol extraction procedure (as described for dopa decarboxylase [3]) is applied to PQQ (conditions are given in section 2.4), PQQ-5,5-dihexyl ketal was formed. If, however, refluxing is allowed to proceed much longer, or a higher HCl concentration is used, the dihexyl ester of this diketal is found (compound 3). Application of the present hexanol extraction procedure (as described under section 2.3) leads to 4-hydroxy-5-hexoxy-PQ in all cases (table 1). The reason why this product is formed under the latter conditions is not clear. In analogy with the production route of 4-hydroxy-PQ from PQQ via PQQH₄ and dehydration [11], reduction should occur. This reduction might be due to impurities in the hexanol used (in agreement with the observation that large amounts of PQQ (more than 50 mg) gave a mixture of 4-hydroxy-5-hexoxy-PQ and PQQ-5,5-dihexyl ketal). The observation that the hemiketal of PQQ

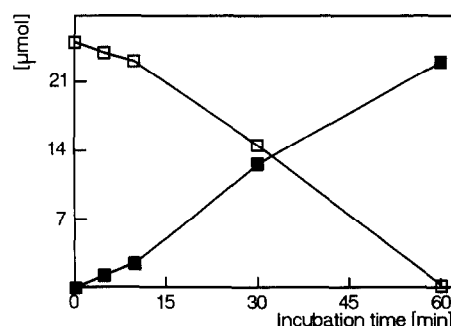


Fig.3. Conversion of 4-hydroxy-5-hexoxy-PQ (compound 1) with cerium ammonium nitrate into PQQ. Incubation of the compounds occurred at room temperature using a ratio as indicated in section 2.6. PQQ (■) and 4-hydroxy-5-hexoxy-PQ (□) were recovered by applying the Sep-Pak procedure and analyzed by HPLC.

Table 1
Results of the hexanol extraction procedure

Enzyme or protein	Amount of protein		PQQ found ^b (nmol)
	Used ^a	Ref.	
BSAO	15.4 nmol	[18]	14.6 (± 1.2)
PKDAO	24.4 nmol	[16]	22.6 (± 0.9)
DBH	10.5 nmol	[19]	40.3 ^c (± 0.8)
MADH	79 nmol	[1]	161 ^d (± 9)
GDC	2.8 nmol	[6]	14.9 ^e (± 0.9)
BSA	1–25 mg		ND
Pronase E	1–10 mg		ND
Glucose oxidase	1–5 mg		ND
Sample	Amount of compound		PQQ found (μ mol)
	Used ^f (μ mol)	Ref.	
PQQ	3.0	[33]	3.0 (± 0.2)
PQQ-acetone	2.5	[33]	2.3 (± 0.3)
PQQH ₂	3.0	[33]	2.9 (± 0.6)
PQQ-oxazole	2.2	[17]	2.0 (± 0.2)
PQQ-DNPH	1.9	[18]	1.8 (± 0.3)
PQQ-Trp	0.8	[17]	0.8 (± 0.2)

^a Based on the M_r and specific absorption coefficient of the native enzyme form (see ref.)

^b Mean value (\pm standard deviation) of three independent determinations (each based on a separate extraction)

^c DBH is a tetrameric enzyme

^d MADH consists of 2 large, and 2 small subunits while the cofactor is present in the small subunits

^e GDC is a hexameric enzyme

^f Based on the molar absorption coefficient (see ref.)

Bovine serum amine oxidase (BSAO), porcine kidney diamine oxidase (PKDAO), dopamine β -hydroxylase (DBH) from bovine adrenal medulla, methylamine dehydrogenase (MADH) from *Thiobacillus versutus*, glutamate decarboxylase (GDC) from *E. coli*, bovine serum albumin (BSA), pronase E, PQQ, the acetone adduct of PQQ, PQQH₂, PQQ-oxazole, the hydrazone of PQQ and 2,4-dinitrophenylhydrazine (PQQ-DNPH), and PQQ-tryptophan (PQQ-Trp) were all subjected to the hexanol extraction procedure described in section 2.3. ND, not detectable

and hexanol is formed immediately when PQQ solutions are mixed with hexanol and acid (results not shown), while formation of the dihexyl ketal under these conditions requires at least 1 h, might indicate that the hemiketal is the precursor.

3.3.2. Enzymes

The results in table 1 show that enzymes proposed to be quinoproteins by the hydrazine method, are also quinoproteins according to the hexanol extraction procedure. Evidence was ob-

tained by comparing the extracted product with authentic 4-hydroxy-5-hexoxy-PQ (by HPLC with photodiode array detection). Moreover, in view of the number of PQQ molecules found per enzyme molecule (table 1), it appears that the methods also give identical quantitative results.

4. DISCUSSION

The original hexanol extraction procedure (as described in [3]), was based on the conversion of the cofactor into free PQQ-5,5-dihexyl ketal. Although it works satisfactory in the case of galactose oxidase [10], it should be realized that the ketal is an inherently labile compound. Moreover, esterification occurs when applied to certain enzymes, leading to a number of peaks in the HPLC chromatogram [3].

The present procedure gave only one product (compound 1) when applied to a variety of enzymes. The structure of this compound is 4-hydroxy-5-hexoxy-PQ, according to comparison with authentic material, and its conversion into PQQ upon oxidation with cerium ammonium nitrate (fig.3). Obviously, the conditions are suitable to transform the cofactor into the stable compound 1 and unsuitable for esterification (also checked with an impure dopa decarboxylase preparation). Moreover, the conversion is quantitative and reproducible, as demonstrated by the results with known amounts of PQQ (below 50 mg) and the fact that the same number of PQQ molecules per enzyme molecule was found as with the hydrazine method (table 1). Therefore, two independent methods based on quite different principles demonstrate that the enzymes mentioned in table 1 are really quinoproteins.

Since the hexanol extraction procedure does not depend on catalytic activity of PQQ, it was interesting to investigate copper-containing amine oxidases. These enzymes contain 2 Cu²⁺ per enzyme molecule but only 1 carbonyl group cofactor, as revealed by careful titration with phenylhydrazine (see e.g. [25] and references therein). Application of the hydrazine method, revealed indeed only 1 covalently bound PQQ per enzyme molecule [16,19]. However, from titration experiments with substrate for bovine plasma amine oxidase purified by a novel method [26], it has been claimed that 2 PQQ's are present [26]. One of the

possibilities which has been put forward to explain this discrepancy is that the enzyme shows half-of-the-sites reactivity for hydrazines [27]. However, this explanation appears to be incorrect since the hexanol extraction procedure also gives 1 PQQ per enzyme molecule. Therefore, the discrepancy could either result from the presence of apo-enzyme in common preparations (a higher specific activity has been reported indeed for the preparations with a higher cofactor-content [26]) or the presence of an additional redox cofactor (it has been claimed by Suva and Abeles [28] that an extra SH group can be detected after substrate addition).

As apparent from table 1, the hexanol extraction procedure is able to convert PQQ and its condensation and addition products into compound 1 in a quantitative way. This finding has important consequences for the analysis of PQQ in mammalian systems, that is in an environment where PQQ is expected to be catalytically inactive (as required by the hydrazine method) and derivatized (covalently bound to a peptide, as oxazole, etc.). Therefore, two preparations were checked in the cases where the hydrazine should fail to detect PQQ. It has been claimed that bovine serum albumin (BSA) contains PQQ [29,30]. However, as apparent from table 1, the preparation used by us did not reveal any PQQ. Perhaps, this discrepancy is related to the finding that commercial preparations of BSA contain variable amounts of copper-containing amine oxidases [31]. The absence of PQQ also applies to pronase E, a proteolytic enzyme mixture which is used in the hydrazine method to detach the adduct from the protein. Although used in the final step (so that it cannot be expected that any hydrazone or azo-compound can be formed), the present result indicates that the hydrazine method can be safely used in cases where it is appropriate as no contamination is introduced by the use of pronase E. Another example where PQQ is absent, is the typical flavoprotein glucose oxidase (table 1).

Finally, it should be mentioned that the hexanol extraction procedure is unable to reveal the precise structure of the cofactor in the active site of methylamine dehydrogenase (MADH) (and perhaps of other quinoproteins). Very recently, X-ray diffraction analysis suggested a structure for the cofactor in this enzyme which has been indicated as pro-PQQ [32]. Pro-PQQ consists of the indole ring of PQQ to which a glutamyl residue is

attached. Apparently, the hydrazine method leads to ring closure since PQQ-phenylhydrazone is obtained when phenylhydrazine is applied to MADH [1]. Obviously, ring closure and oxidation also occurs with the hexanol extraction procedure since the extracted product was chromatographically and spectrally identical to authentic compound 1. Therefore, although the hexanol extraction procedure has the potentials required for analysis of derivatized PQQ, a more delicate analysis procedure should be developed to study the structure of the cofactor of enzymes, presently indicated as quinoproteins with covalently bound PQQ.

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REFERENCES

- [1] Van der Meer, R.A., Jongejan, J.A. and Duine, J.A. (1987) *FEBS Lett.* 221, 299-304.
- [2] Duine, J.A. and Jongejan, J.A. (1989) *Annu. Rev. Biochem.* 58, 403-426.
- [3] Groen, B.W., Van der Meer, R.A. and Duine, J.A. (1988) *FEBS Lett.* 237, 98-102.
- [4] Van Kleef, M.A.G. and Duine, J.A. (1988) *FEBS Lett.* 237, 91-97.
- [5] Houck, D.R., Hanners, J.L. and Unkefer, C.J. (1988) *J. Am. Chem. Soc.* 110, 6920-6921.
- [6] Van der Meer, R.A., Groen, B.W. and Duine, J.A. (1989) *FEBS Lett.* 246, 109-112.
- [7] Van Kleef, M.A.G. and Duine, J.A. (1988) *BioFactors* 1, 297-302.
- [8] Matsumoto, T., Suzuki, O., Hayakawa, H., Ogiso, S., Hayakawa, N., Nimura, Y., Takahashi, I. and Shionoya, S. (1989) in: *Proceedings of the first international symposium on PQQ and quinoproteins* (Jongejan, J.A. and Duine, J.A. eds) pp. 162-164, Kluwer Academic Publishers, Dordrecht; Rucker, R., Killgore, J., Duich, L., Romero-Chapman, N., Smidt, C. and Tinker, D. (1989) in: *Proceedings of the first international symposium on PQQ and quinoproteins* (Jongejan, J.A. and Duine, J.A. eds) pp. 159-161, Kluwer Academic Publishers, Dordrecht; Rucker, R., Killgore, J., Duich, L., Romero-Chapman, N., Smidt, C. and Tinker, D. (1989) in: *Proceedings of the first international symposium on PQQ and quinoproteins* (Jongejan, J.A. and Duine, J.A., eds) pp. 159-161, Kluwer Academic Publishers, Dordrecht.
- [9] Van Kleef, M.A.G., Jongejan, J.A. and Duine, J.A. (1989) *Eur. J. Biochem.*, in press.
- [10] Van der Meer, R.A., Jongejan, J.A. and Duine, J.A. (1989) *J. Biol. Chem.* 264, 7792-7794.
- [11] Frank, J., Dijkstra, M., Balny, C., Verwiël, P.E.J. and Duine, J.A. (1989) *Eur. J. Biochem.*, in press.

- [12] Corey, E.J. and Tramontano, A. (1981) *J. Am. Chem. Soc.* 103, 5599-5600.
- [13] Jongejan, J.A. (1989) PhD Thesis, Delft University of Technology.
- [14] Duine, J.A. and Frank, J. (1980) *Biochem. J.* 187, 221-226.
- [15] Duine, J.A., Frank, J. and Verwiël, P.E.J. (1980) *Eur. J. Biochem.* 108, 187-192.
- [16] Van der Meer, R.A., Jongejan, J.A., Frank, J. and Duine, J.A. (1986) *FEBS Lett.* 206, 111-114.
- [17] Van Kleef, M.A.G., Jongejan, J.A. and Duine, J.A. (1989) in: *Proceedings of the first international symposium on PQQ and quinoproteins* (Jongejan, J.A. and Duine, J.A. eds) pp. 217-226, Kluwer Academic Publishers, Dordrecht.
- [18] Lobenstein-Verbeek, C.L., Jongejan, J.A., Frank, J. and Duine, J.A. (1984) *FEBS Lett.* 170, 305-309.
- [19] Van der Meer, R.A., Jongejan, J.A. and Duine, J.A. (1988) *FEBS Lett.* 231, 303-307.
- [20] Snijder, C. and Rapoport, H. (1972) *J. Am. Chem. Soc.* 94, 227.
- [21] Dekker, R.H., Duine, J.A., Frank, J. and Verwiël, P.E.J. (1982) *Eur. J. Biochem.* 125, 69-73.
- [22] Salisbury, S.A., Forrest, H.S., Cruse, W.B.T. and Kennard, O. (1979) *Nature* 280, 843-844.
- [23] Itoh, S. and Ohshiro, Y. (1986) *Chem. Express* 1, 315-318.
- [24] Duine, J.A., Frank, J. and Verwiël, P.E.J. (1981) *Eur. J. Biochem.* 118, 395-399.
- [25] Falk, M.C. (1983) *Biochemistry* 22, 3740-3745.
- [26] Hartmann, C. and Klinman, J.P. (1987) *J. Biol. Chem.* 262, 962-965.
- [27] Van der Meer, R.A., Jongejan, J.A. and Duine, J.A. (1989) in: *Proceedings of the first international symposium on PQQ and quinoproteins* (Jongejan, J.A. and Duine, J.A. eds) pp. 111-122, Kluwer Academic Publishers, Dordrecht.
- [28] Suva, R.H. and Abeles, R.H. (1978) *Biochemistry* 17, 3538-3545.
- [29] Paz, M.A., Gallop, P.M., Torrelío, B.M. and Flueckiger, R. (1988) *Biochem. Biophys. Res. Commun.* 154, 1330-1337.
- [30] Adachi, O., Shinagawa, E., Matsushita, K., Nakashima, K., Takimoto, K. and Ameyama, M. (1989) in: *Proceedings of the first international symposium on PQQ and quinoproteins* (Jongejan, J.A. and Duine, J.A. eds) pp. 145-147, Kluwer Academic Publishers, Dordrecht.
- [31] Al-Naji, A.S. and Clarke, D.E. (1983) *Life Sci.* 32, 635-643.
- [32] Vellieux, F.M.D., Huitema, F., Groendijk, H., Kalk, K.H., Frank, J., Jongejan, J.A., Duine, J.A., Drenth, J. and Hol, W.G.J. (1989) *EMBO J.*, in press.
- [33] Duine, J.A., Frank, J.A. and Jongejan, J.A. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol.* 59, 169-212.